DNA Methylation Regulates Corneal Epithelial Wound Healing by Targeting miR-200a and CDKN2B

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PURPOSE. DNA methylation is a key epigenetic modification involved in various biological processes and diseases. Corneal epithelial wound healing (CEWH) is essential for restoring corneal integrity and transparency after injury. However, the role of DNA methylation in CEWH remains elusive. Here, we investigate the function and underlying mechanism of DNA methylation in regulating CEWH.

METHODS. Dot blots and global methylation assays determined DNA methylation levels during CEWH. Quantitative RT-PCR and Western blot analysis examined the expression of DNA methyltransferase (DNMTs), cyclin-dependent kinase inhibitor 2B (CDKN2B), and miR-200a during CEWH, respectively. MTS assays and flow cytometry were used to analyze human corneal epithelial cell (HCEC) proliferation and cell cycle, respectively. The in vitro scratch wound assay assessed HCEC migration and an in vivo murine corneal epithelial debridement model evaluated wound healing. Using bisulfite sequencing PCR, we determined the DNA methylation status of the candidate genes. Transfection of miR-200a mimic or inhibitor assessed the function of miR-200a in HCECs. Rescue experiments were performed to clarify the correlation between DNMT1 and miR-200a/CDKN2B during CEWH.

RESULTS. DNMT1 and DNMT3B expression was significantly upregulated during CEWH, resulting in a significant global DNA hypermethylation. DNMT1 downregulation dramatically delayed CEWH in vivo, and suppressed HCEC proliferation and migration. MiR-200a inhibited HCEC migration. Furthermore, miR-200a and CDKN2B were identified as molecular targets of DNA methylation and as having a causal connection with DNMT1.

CONCLUSIONS. DNMT1-mediated DNA hypermethylation can enhance the process of CEWH by directly targeting miR-200a and CDKN2B. This insight pinpoints novel potential drug targets for promoting CEWH.

Keywords: DNA methylation, corneal epithelial wound healing, miR-200a, CDKN2B

Corneal re-epithelialization after injury is essential for prevention of stromal pathogenic infiltration, which often leads to corneal opacification and loss of tissue integrity. This response reduces the likelihood of infection by restoring an intact epithelial layer and tight junctional barrier function. Several drugs are known to promote epithelial healing; however, these drugs often have side effects that compromise corneal function. Although it is well known that the methylation status of DNA affects gene expression patterns, there are still no reports describing the effects of DNA methylation alterations on corneal epithelial wound healing (CEWH). A better understanding of this mechanism will help identify novel gene targets and develop new therapeutics that promote CEWH.

DNA methylation is an essential epigenetic mechanism in regulating gene expression and maintaining genomic integrity. This process is driven by three different DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B. DNMTs catalytically methylate the C-5 ring position on the cytosine residues of enriched CpG dinucleotides in the promoter region of many genes. A number of studies indicate that enzyme-mediated DNA methylation affects cell proliferation, cell migration, cell differentiation, development, and aging. Aberrant DNA methylation has been implicated in numerous human diseases, such as autoimmune diseases, neurodegenerative disorders, and cancer, as well as ocular diseases. MicroRNAs (miRNAs) constitute another epigenetic mechanism controlling posttranscriptional gene expression by mediating either mRNA degradation or blocking translation in a sequence-specific manner. We previously identified 29 miRNAs that had a significant change in expression during CEWH. Among this group, the levels of miR-204, miR-200a, and miR-200b were markedly decreased. Alterations in these miRNA expression patterns are relevant to the CEWH process, as their gene targets are essential for promoting cell migration and proliferation. The underlying mechanisms that mediate control of miRNA expression are not well understood. Changes in the DNA methylation status of specific genes may contribute to the process that modulates the response to injury. As an increase in cell proliferation is accompanied by alterations in cyclin-dependent kinase inhibitor (CDKN) 1A, CDKN1B, and CDKN2B expression during CEWH, an association was sought between such effects and changes in DNA methylation status. There is an indication that such an association exists.
because CDKNs were silenced by promoter hypermethylation in a variety of tumors. However, it remains unclear whether these CDKNs are affected by changes in DNA methylation status during CEWH.

Here, we show that corneal epithelial injury induces an increased DNMT1 and DNMT3B expression levels and this is accompanied by significant global DNA hypermethylation. DNMT1 downregulation significantly decreased the rate of CEWH in vivo and suppressed human corneal epithelial cell (HCEC) proliferation and migration. The underlying mechanism involves decreased miR-200a and CDKN2B (HCEC) proliferation and migration. These findings could help better understand epigenetic regulation during CEWH and identify novel potential drug targets to promote CEWH.

**Materials and Methods**

**Animals and CEWH Model**

Eight-week-old C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Animals were housed under climate-controlled conditions with a 12-hour light/dark cycle and provided with ad libitum laboratory diets. In vivo mouse models of CEWH were established as previously described. Antibodies for DNMT1, DNMT3A, DNMT3B, and β-actin were purchased from Cell Signaling Technology. The enhanced chemiluminescence method was used to detect immunoreactive bands. To ensure protein loading equivalence, blots were probed with a β-actin antibody. Densitometry of the Western blot protein bands was analyzed using AlphaView FluorChemQ (ProteinSimple, San Jose, CA, USA).

**Global Methylation Assay**

Genomic DNA from corneal epithelia was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Genomic DNA content was quantified by the Nanodrop One (Thermo Fisher Scientific, Waltham, MA, USA). The global DNA methylation status of the corneal epithelia was determined using the MethylFlash Global DNA Methylation ELISA Easy Kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, 100 ng genomic DNA was bound to each well. After washing, an aliquot of 5-methylcytosine (5-mC) detection complex solution was added and incubated at room temperature for 30 minutes. After washing again, the developer solution was added and the optical absorbance was measured using a microplate reader at 450 nm. The percentage of methylated DNA was calculated based on linear regression analysis.

**Dot Blots**

As previously described, DNA samples were denatured at 99°C for 5 minutes, and 2-fold serial dilutions of DNA samples were spotted on nylon membranes. The nylon membranes were then baked at 65°C for 15 minutes and blocked with 5% skim milk at room temperature for 30 minutes. The membranes were incubated with primary antibody (anti-5mC; 1:1000 dilution; Active Motif, Carlsbad, CA, USA) at 4°C overnight. After incubation with an appropriate secondary antibody (1:3000 dilution; Cell Signaling Technology, Beverly, MA, USA), autoradiogram images were acquired and analyzed using AlphaView Fluor ChemQ (ProteinSimple, San Jose, CA, USA).

**Quantitative RT-PCR**

Total RNA was isolated from the corneal epithelia using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR (RT-qPCR) assays were performed on the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) using Taqman mRNA assay kit or Taqman microRNA assay kit (Thermo Fisher Scientific). β-actin or U6 was used as an endogenous control to normalize the relative expression of the target gene. Data were analyzed and expressed as relative gene expression using the 2-DDCt method.

**Western Blot**

RIPA lysis buffer was used to extract corneal epithelial total protein. Western blot analysis was performed as previously reported. Antibodies for DNMT1, DNMT3A, DNMT3B, and β-actin were purchased from Cell Signaling Technology. The enhanced chemiluminescence method was used to detect immunoreactive bands. To ensure protein loading equivalence, blots were probed with a β-actin antibody. Densitometry of the Western blot protein bands was analyzed using AlphaView FluorChemQ (ProteinSimple). β-actin was used as an endogenous control to evaluate the relative expression of the target protein.

**Intrastromal Injection of Small Interfering RNA (siRNA) and Monitoring of Wound Healing**

Corneal epithelial gene knockdown was performed by intrastromal siRNA injection, which has been previously used for gene delivery into the corneal epithelia. A slightly modified intrastromal injection method was used to downregulate target genes. All injections were performed under a dissection microscope (Nikon, Garden, NY, USA). Mice were anesthetized with an intraperitoneal injection of xylazine (15 mg/kg) and ketamine (87 mg/kg), as well as topical treatment of oxybuprocaine. The head of the anesthetized mouse was stabilized on a Styrofoam platform and the eye was proptosed with forceps. A total volume of 3 µl polyethylenimine (PEI; Polyplus-transfection, New York, NY, USA)-complexed siDnmt1 (0.1 mM), siDnmt3b (0.1 mM), or negative control (NC) was injected into the midperipheral cornea extending into the anterior stroma using a 33-gauge needle with a bevel of 30° (Hamilton, Bonaduz, Switzerland). The siRNA was injected under the entire surface of the corneal epithelia, and the characteristic transient whitening of the cornea was observed. The CEWH assay was performed 6 hours after intrastromal siRNA injection. The wound healing rate was monitored by staining the corneal epithelia with fluorescein and quantified at 0 and 24 hours. At 24 hours, the corneal epithelia were isolated to quantify Dnmt1, Dnmt3b, and Cdkn2b mRNA or the corresponding protein expression levels. The in vivo rescue experiments entailed injecting a total volume of 3 µl PEI-complexed siRNAs as follows: (1) siDnmt1 (0.1 mM) plus NC (0.1 mM); (2) siDnmt1 (0.1 mM) plus siCdkn2b (0.1 mM); (3) siDnmt1 (0.1 mM) plus miR-200a inhibitor (0.1 mM); or (4) siDnmt1 (0.1 mM) plus siCdkn2b (0.1 mM) plus miR-200a inhibitor (0.1 mM).

**Cell Culture**

As previously described, SV40-immortalized HCECs (gift from Araki Sasaki Kagoshima, Miyata Eye Clinic, Kagoshima, Japan)
were cultured in supplemented Dulbecco’s modified Eagle’s medium (DMEM/F12; Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO₂.

Cell Proliferation Assay
As previously described, 3000 cells were plated in each well of a 96-well plate (Corning, Inc., Corning, NY, USA). After 24-hour culture, transfections were carried out using Lipofectamine RNAiMAX (Invitrogen). Cell proliferation was assessed using the MTS assay kit (CellTiter 96 Aqueous; Promega Corp., Madison, WI, USA) according to manufacturer’s instructions.

Flow Cytometry Analysis
As previously described, HCECs (1 × 10⁵) were stained with propidium iodide after 48-hour transfection according to manufacturer’s instructions. DNA content was analyzed with a flow cytometer (FACS caliber, Becton Dickinson, San Jose, CA, USA).

Scratch Wound Assay
As previously described, HCECs (1.2 × 10⁵) were plated in each well of a 12-well plate (Corning, Inc.). Transfections were performed using Lipofectamine RNAiMAX with either siDNMT1, a miR-200a mimic, a miR-200a inhibitor, siDNMT1 plus NC, or siDNMT1 plus miR-200a inhibitor into HCECs at 24 hours after culture. After 48 hours, a 100 μL sterilized pipette tip was used to make a “scratch” of the epithelial cell surface layer. Then 1 mL fresh serum-free medium was added. After 24 hours, extent of wound closure was evaluated based on photographs of the cultures (Imager Z1; Zeiss, Jena, Germany). Wound healing areas were measured by ImageJ software (1.46r; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The initial cell-free area is designated A₀, and the cell-free area remaining after 24 hours is identified as A₂₄. Wound healing areas were calculated by subtracting A₂₄ from A₀, and wound healing is reported as a percentage = (A₀ − A₂₄) / A₀ × 100%.

Bisulfite Sequencing (BSP) PCR
As previously described, using the UCSC Genome Browser the promoter sequences of miR-204, miR-200a, miR-200b, Cdkn1a, Cdkn1b, and Cdkn2b were identified. BSP primers for the identified promotor sequences were designed using MethPrimer software (Supplementary Table S1). Bisulfite conversion of genomic DNA extracted from the corneal epithelia was performed with an EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA, USA). The bisulfite-treated DNA samples were then subjected to PCR amplification. PCR products were cloned using a ZeroBack Fast Ligation Kit (Tsingke, Beijing, China) and sequenced. Methylation of CpG sites was analyzed using the quantification tool for methylation analysis (QUMA).

Statistical Analysis
All data were analyzed using 2-tailed t-tests to determine the statistical significance between the control group and the WH group, or between the NC group and the siRNA-treated group. P values less than 0.05 were considered significant. All data are shown as mean ± SEM. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
RESULTS

Global DNA Hypermethylation Occurs During CEWH

To ascertain the role of DNA methylation in CEWH, we first determined the global DNA methylation levels at 48 hours after corneal epithelial wounding. Global epithelial DNA methylation levels were significantly increased as 5mC% was elevated in the WH group compared to the control group (Fig. 1A). Additionally, dot blot analysis showed that 5mC% was significantly higher in the healing corneal epithelial cells than in their noninjured control counterparts (Fig. 1B). Together these data suggest that CEWH is associated with increased global DNA methylation levels. We then evaluated the time-dependent changes in DNA methylation levels during CEWH. As shown in Figure 1C, the global DNA methylation level was significantly increased at 12 hours after corneal epithelial wounding and reached a maximum at 48 hours. Subsequently, DNA methylation progressively decreased until the values were restored to control levels at 96 hours (Fig. 1C).

DNMT1 and DNMT3B Expression Levels Are Upregulated During CEWH

As DNA methylation is catalyzed by the DNMT family,4 we determined which subfamily member mediates global DNA hypermethylation during CEWH. The expression levels of DNMT1, DNMT3A, and DNMT3B were measured with both RT-qPCR and Western blot analysis at 48 hours after corneal epithelial injury. As shown in Figure 2, DNMT1 and DNMT3B expression levels significantly increased in the WH groups, whereas DNMT3A expression did not change compared to the control group.

DNA Methylation Promotes Corneal Wound Healing

Figure 3. In vivo knockdown of Dnmt1 delays CEWH. (A) Representative images of fluorescein staining showed that corneas with Dnmt1 siRNA injection have larger wounds 24 hours after injury compared to the negative control (NC)-treated corneas. Wound edges are demarcated in red. (B) Quantification of the area of the wound in the NC- and Dnmt1 siRNA-treated groups 24 hours later was performed (n = 5/group). (C) RT-qPCR showing difference in the Dnmt1 gene expression levels between the NC- and the Dnmt1 siRNA-treated groups (n = 5/group). (D) Western blot images show protein levels of DNMT1 and β-actin. (E) Densitometric Western blot analysis showing a difference in the DNMT1 protein levels between the NC- and the Dnmt1 siRNA-treated groups (n = 5/group). (F) Dot blot analysis of 5-mC levels in the NC- and the Dnmt1 siRNA-treated groups (n = 4/group). (G) Quantification of the 5-mC content in DNA of corneal epithelia isolated from the NC- and the Dnmt1 siRNA-treated groups (n = 4/group).
In Vivo Knockdown of Dnmt1 Retards CEWH

To determine the role of upregulated DNMT1 and DNMT3B in CEWH, the gene expression of Dnmt1 or Dnmt3b was silenced. To accomplish this, corneas were injected with either Dnmt1 siRNA or Dnmt3b siRNA in vivo. As shown in Figure 3A, the wounded area of Dnmt1 siRNA-injected corneas is significantly larger than in NC-injected corneas (Fig. 3B). This difference can be attributed to the siRNA-mediated silencing of Dnmt1 in the corneal epithelium at both the mRNA and protein levels (Figs. 3C–E). By contrast, in vivo CEWH was not significantly different with Dnmt3b siRNA treatment compared to the NC group (Supplementary Fig. S1). To clarify the link between DNMT1 expression and DNA methylation, the effect of in vivo siDnmt1 injection was determined on global DNA methylation levels. Dot blot analysis and global methylation assays showed that 5mC% was significantly decreased in the Dnmt1 siRNA-injected corneal epithelium compared to NC-injected corneal epithelium (Figs. 3F, 3G).

Figure 4. DNMT1 loss of function suppresses proliferation and migration of HCECs. (A) Western blots confirmed a decrease in DNMT1 protein expression. β-actin was used as a normalization protein. DNMT1 loss of function was characterized 48 hours after HCEC transfection with DNMT1 siRNA. NC designates negative control–transfected HCECs. (B) Dot blot analysis of 5mC levels in the NC- and the DNMT1 siRNA-transfected cells (n = 2/group). (C) MTS assays were performed to compare the proliferation rates between NC- and DNMT1 siRNA-transfected HCECs (n = 6/group). (D) HCECs were treated with siDnmt1 or NC for 48 hours and examined using flow cytometric analysis to determine the cell cycle phase distribution. Inserts show the distribution of cells expressed as percentages in different phases of the cell cycle. (E) The scratch wound assay was used to characterize HCEC migration in NC- and DNMT1 siRNA-transfected groups. (F) Quantification of wound healing in NC- and DNMT1 siRNA-transfected cells using the scratch wound assay (n = 3/group).

Figure 5. DNA hypermethylation suppresses miR-200a gene expression during CEWH. (A) Quantitative analysis of miR-200a gene expression in control and WH corneal epithelia (n = 3/group). (B) The position of the CpG sites at the upstream regulatory genomic locus of miR-200a. Analysis of DNA methylation in the mouse miR-200a locus in control and WH corneal epithelia by BSP. TSS: transcriptional start site. (C) Global percent methylation of all clones from the control and WH corneal epithelia (n = 8/group).
Downregulation of DNMT1 Inhibits HCEC Proliferation and Migration

To assess the functional role of DNMT1 in mediating CEWH, we used specific siRNA to knock down its expression in HCECs. The siRNA transfection efficiency was confirmed using fluorescence microscopy of Cy3-labeled siRNA (Supplementary Fig. S2A). Our results indicate that downregulation of DNMT1 is accompanied by a dramatic decrease in global DNA methylation (Figs. 4A, 4B). HCECs transfected with DNMT1 siRNA had a significant decrease in cell proliferation (Fig. 4C). Consistent with these results, the distribution of cells in the cell cycle S phase increased with DNMT1 siRNA transfection compared to NC-transfected HCECs (Fig. 4D). Such effect was further confirmed by an EdU proliferation assay (Supplementary Figs. S2B, S2C). However, knockdown of DNMT1 did not induce apoptosis (Supplementary Figs. S2D, S2E).

To determine the role of DNMT1 in cell migration of HCECs, we performed the in vitro scratch wound assay. Cultures of HCECs transfected with DNMT1 siRNA had larger wound areas after 24 hours, indicating that cell migration was significantly decreased compared to NC-transfected HCECs (Figs. 4E, 4F).

MiR-200a and CDKN2B Are Targets of DNA Methylation During CEWH

Having established an association between DNA hypermethylation and CEWH, we next sought to understand the underlying mechanism that contributes to this response. We previously reported that 15 miRNAs were dramatically downregulated during CEWH.14 As it is known that DNA hypermethylation inhibits gene expression,4 we aimed to determine the effect of DNA hypermethylation on miRNA expression in CEWH. As seen in Figure 5A, miR-200a expression is significantly decreased during CEWH (Fig. 5A). In five CpG-rich upstream regulatory regions, the BSP assay results indicated that 42.5% of the CpG sites were methylated in the control group compared to 88.9% of sites in the WH group (Fig. 5B). The increase in DNA methylation occurred in the upstream miR-200a regulatory region that spans from −832 to −592 nucleotides (nt (Fig. 5C). By contrast, no significant changes were observed in the methylation within the upstream regulatory regions of miR-204 (Supplementary Fig. S3A) or miR-200b (Supplementary Fig. S3B). We next verified if in vivo knockdown of Dnmt1 reduces methylation. As expected, the BSP assay results showed that 87.1% of the CpG sites were methylated in the NC group compared to 58.6% in the WH group (Supplementary Fig. S4A). Significant miR-200a hypomethylation was observed in the upstream regulatory region spanning from −832 to −592 nt (Supplementary Fig. S4B); miR-200a expression was significantly increased in the siDnmt1-injected corneas compared to that of the NC group (Supplementary Fig. S4C).

CDKN2B is a well-known negative regulator of the cell cycle progression.18,19 This led us to examine Cdkn2b Cpg methylation status. The fact that DNA hypermethylation frequently occurs in regulatory upstream regions in cancer cells19,20 suggests that Cdkn2b may undergo similar DNA methylation pattern during CEWH. Our results indicate that Cdkn2b gene and protein expression levels were downregulated during CEWH (Figs. 6A–C). In six CpG-rich upstream regulatory regions, BSP assay results indicated that 25.9% of the CpG sites were methylated in the control group compared to 74.1% in the WH group (Fig. 6D). Significant Cdkn2b hypermethylation was observed in the upstream regulatory region.

**Figure 6.** DNA hypermethylation suppresses Cdkn2b gene expression during CEWH. (A) Quantitative analysis of Cdkn2b gene expression in control and WH corneal epithelia (n = 3/group). (B) Western blot analysis was used to assess the levels of CDKN2B and β-actin protein expression in control and WH corneal epithelia. (C) Densitometric Western blot analysis indicated significantly decreased CDKN2B protein expression (n = 3/group). (D) The position of the CpG sites at the upstream genomic locus of Cdkn2b. Analysis of DNA methylation status at the upstream regulatory locus of the mouse Cdkn2b gene in control and WH groups determined by BSP. TSS: transcriptional start site. (E) Global percentage of methylation of all clones from the control and WH corneal epithelia (n = 18/group).
that spans from $-1468$ to $-1299$ nt (Fig. 6E). By contrast, a difference in the methylation status in the same upstream region of Cdkn1a or Cdkn1b was not observed between the control and WH groups (Supplementary Fig. S5). Similarly, we also confirmed that knockdown of Dnmt1 causes promoter hypomethylation and increases expression of Cdkn2b in vivo. In six CpG-rich upstream regulatory regions, the BSP assay results showed that 76.9% of the CpG sites were methylated in the NC group compared to 58.3% in the WH group (Supplementary Fig. S6A). Significant Cdkn2b hypomethylation was seen in the upstream regulatory region spanning from $-1468$ to $-1299$ nt (Supplementary Fig. S6B). CDKN2B expression was significantly increased in the siDnmt1-injected corneas compared to that of the NC group ( Supplementary Figs. S6C–E).

MiR-200a Inhibits HCEC Migration

To confirm that the decrease in miR-200a expression contributes to an increase in HCEC migration after injury, we performed experiments that silenced gene expression of miR-200a or miR-200a gain-of-function experiments. To examine the effect of the loss of miR-200a, HCECs were transfected with a miR-200a inhibitor. Using MTS assays and the scratch wound assay, we did not observe a change in cell proliferation (data not shown), but saw an increase in cell migration with miR-200a inhibition (Figs. 7A, 7B). By contrast, cell migration was decreased in miR-200a mimic–transfected HCECs (Figs. 7C, 7D). These data suggest a reciprocal relationship between a decrease in miR-200a expression and an increase in corneal epithelial migration during CEWH.

Rescue Experiments Confirm a Causal Connection Between DNMT1 and miR-200a/CDKN2B During CEWH

To firmly establish a direct causal relationship between DNMT1 and miR-200a and/or CDKN2B in DNMT1-mediated regulation of CEWH, we performed both in vitro and in vivo rescue experiments. In an in vitro rescue experiment, we transfected siDNMT1 plus miR-200a inhibitor into HCECs to determine the effect on cell migration in the scratch wound assay. Small interfering DNMT1–mediated upregulation of miR-200a and CDKN2B in HCECs was confirmed by RT-qPCR and Western blot analysis (Supplementary Fig. S7). As shown in Figures 8A and 8B, cotransfection of siDNMT1 and miR-200a inhibitor significantly increased cell migration. We also cotransfected siDNMT1 and siCDKN2B into HCECs to evaluate their effects on cell proliferation with flow cytometry, MTS, and EdU assays. In siDNMT1- plus siCDKN2B-transfected HCECs, cell proliferation was also significantly increased compared to the control group (Fig. 8C, Supplementary Fig. S8). Consistent with these results, the distribution of cells in S phase decreased in siDNMT1- plus siCDKN2B-transfected HCECs (Fig. 8D).

In vivo rescue experiments, we injected either a combination of siDnmt1 plus siCdkn2b, siDnmt1 plus miR-200a inhibitor, or all three siRNAs into the mouse corneas to assess their effects on CEWH. As shown in Figure 9A, the wound area at 24 hours post injury in the siDnmt1- plus
siCdkn2b-injected corneas was significantly smaller than in siDnmt1- plus NC-injected corneas (Fig. 9B). Corneas injected with siDnmt1 plus miR-200a inhibitor appeared to have smaller unhealed areas compared to siDnmt1- plus NC-injected corneas (marginally significant, \( P = 0.07 \); Figs. 9C, 9D). Additionally, the wound area in corneas injected with all three siRNAs (siDnmt1 plus siCdkn2b plus miR-200a inhibitor) was significantly smaller compared to siDnmt1- plus NC-injected corneas (Figs. 9E, 9F).

In summary, the inhibition of miR-200a prevents the DNMT1-mediated decrease of cell migration. Similarly, suppression of CDKN2B rescues the DNMT1-mediated decrease in cell proliferation. Furthermore, siDnmt1-mediated delayed CEWH was rescued in vivo by either Cdkn2b suppression or miR-200a/Cdkn2b corepression. Overall, our data establish a direct causal link between DNMT1 and miR-200a/CDKN2B in DNMT1-mediated regulation of CEWH.

**DISCUSSION**

Emerging evidence implies that epigenetic mechanisms, such as miRNAs, can modulate CEWH.\(^{14,23}\) The DNA methylation status of specific genes was first suggested as an epigenetic mark in mediating pathophysiological responses underlying disease.\(^{24}\) However, it was not understood if changes in the DNA methylation affect CEWH. Here, we show that global DNA methylation levels significantly increased during CEWH due to the upregulation of DNMT1 and DNMT3B expression. DNMT subfamily members contribute to the control of cell proliferation, migration, and differentiation in health and various pathological conditions by increasing DNA methylation levels.\(^{25,26}\) Accordingly, we sought to determine if DNA methylation is involved in controlling cell proliferation and migration during CEWH, as increased DNA methylation has been implicated in metastatic diseases.\(^{27}\) Furthermore, understanding the role of DNA methylation in corneal physiological responses is important as abnormal DNA methylation patterns have been implicated in corneal endothelial pathologies that result in loss of corneal transparency.\(^{28}\)

The selective effect of Dnmt1 and Dnmt3b gene silencing made it possible to determine individual contributions to CEWH. Interestingly, although two DNMT subfamily members were upregulated, only Dnmt1 downregulation inhibited CEWH in vivo. Similarly, our in vitro results demonstrated that siRNA-mediated DNMT1 downregulation inhibited HCEC proliferation and migration. It is noteworthy that although the Dnmt3b expression level increased during CEWH, silencing it did not impede this process. Nevertheless, DNMT3B is thought to be essential for establishing the de novo DNA methylation patterns during early development.\(^{29}\) DNMT1 has been mainly implicated in maintaining methylation patterns associated with increases in both DNA replication and cell proliferation.\(^{30}\) It is well known that DNMT1 and DNMT3B play dichotomous roles in control of DNA methylation status. However, some reports suggest that DNMT1 and DNMT3B have overlapping effects in some processes.\(^{31,32}\) Although Dnmt3b knockdown failed to inhibit CEWH in vivo, it is possible that the effect of DNMT3B synergizes with the increase in cell proliferation and migration mediated by DNMT1. Another possibility is that DNMT3B is involved in a different process in CEWH that we did not explore. Future studies are needed to clarify the triggering mechanisms that
increase DNMT1 and DNMT3B expression levels, and further examine if DNMT3B downregulation participates in controlling other responses involved in CEWH.

It is important to note that alteration of DNA methylation on a genome-wide scale is far less frequent than it is on individual genes in physiological processes and diseases. During early mammalian development, there are at least two dynamic changes in global DNA methylation associated with erasing cellular memory and creating cellular diversity.33 For most cancer types, it is well known that a decrease in global DNA methylation is a common feature in tumorigenesis.34 In addition, global DNA methylation is also a potential biomarker in several obesity-related diseases such as diabetes, cardiovascular disease, and metabolic syndrome.35–37 To the best of our knowledge, our study is the first to explore the role of DNA methylation in CEWH. Our study also reinforces the notion that epigenetic changes can serve as signature of CEWH. Therefore, upregulation of DNA methylation is a promising strategy to promote CEWH, and DNMT1 may be a potential drug target.

**FIGURE 9.** In vivo rescue experiments reveal a direct causal correlation between Dnmt1 and miR-200a/Cdkn2b. (A) Representative images of fluorescein staining showed that corneas injected with complexes of siDnmt1 plus siCdkn2b have smaller unhealed areas after 24 hours than the siDnmt1- plus NC-treated corneas. Wound edges are demarcated in red. (B) Quantification of the healed area with siDnmt1- plus NC- or siDnmt1- plus siCdkn2b-treated groups at 24 hours (n = 5/group). (C) Representative images of fluorescein staining showed that corneas transfected with siDnmt1 plus miR-200a inhibitor have smaller unhealed areas after 24 hours than the siDnmt1- plus NC-treated corneas. Wound edges are demarcated in red. (D) Quantification of the healed area with siDnmt1 plus NC or siDnmt1 plus miR-200a inhibitor treatment at 24 hours (n = 6/group). (E) Representative images of fluorescein staining showed that corneas transfected with siDnmt1 plus siCdkn2b plus miR-200a inhibitor have smaller unhealed areas at 24 hours compared to corneas treated with the siDnmt1 plus NC. Wound edges are demarcated in red. (F) Quantification of the healed area with siDnmt1 plus NC, and siDnmt1 plus siCdkn2b plus miR-200a inhibitor treatment at 24 hours (n = 7/group).
healing that occurs in other tissues, such as skin, as there may be similar shared mechanisms.38,39

Although the DNA methylation levels vary in different tissues, the DNA methylation status of specific gene promoters in different individuals is the same.38 Increasing evidence suggests that epigenetic silencing of miRNA gene expression by CpG hypermethylation is also emerging as a common hallmark of physiological development and specific diseases.41-43 Generally, DNA methylation occurs in only approximately 1.5% of the human genome;43 however, it regulates multiple targets ranging from protein-coding genes to non-coding genes.44 Our previous studies demonstrated that miR-204, miR-200a, and miR-200b expression levels were remarkably decreased during CEWH.14 These findings prompted us to examine changes in miRNA gene methylation status in their upstream promoter regions during CEWH. Consequently, we found that only miR-200a downregulation was associated with a hypermethylation in its upstream promoter regulatory region. Similarly, a previous study demonstrated that the downregulation of miR-200a promotes an epithelial-to-mesenchymal transition (EMT)-like transdifferentiation in mammary epithelial cells.45 Therefore, increased DNA methylation in the miR-200a promoter region leads to miR-200a gene silencing contributing to the acceleration of CEWH.

CDKNs are inhibitors of cell cycle progression, and changes in their expression modulate cell cycle progression and rates of Cep140 hypermethylation of cell cycle regulatory genes.46 As push–pull interaction between cyclin-dependent kinases (CDK) and CDKNs, this is possibly modulated through changes in DNA methylation.48,49 We confirmed that the increase in cell proliferation that occurs during CEWH is a consequence of CDKN2B downregulation mediated by an increase in DNA methylation of its gene promoter region. Thus, the increase in cell proliferation that is the result of decreased CDKN2B expression agrees with the argument for a push–pull interaction between CDK6 and CDKN2B.48 Moreover, previous studies strongly suggest that CDKN2B upregulation inhibits epithelial cell proliferation in the cornea and in other tissues.48,49

Hypermethylation of the promoters of miR-200a and CDKN2B decreased their expression levels and contributed to the regulation of CEWH. By contrast, hypomethylation of the same region has the opposite effect. These data, together with both in vivo and in vitro rescue experiments, established a direct causal relationship between DNMT1 and miR-200a.48 CDKN2B. However, considering the complexity of regulatory mechanisms controlling CEWH, we do not exclude other possible regulatory mechanisms in addition to DNMT1. Nevertheless, our data clearly demonstrate that DNMT1-mediated regulation of CDKN2B and miR-200a, at least in part, is responsible for the functional changes caused by DNA methylation during CEWH.

In summary, DNMT1-mediated DNA hypermethylation facilitates CEWH via downregulation of miR-200a and CDKN2B leading to changes in cell proliferation and migration. Our findings provide a better understanding of the epigenetic regulation that occurs during CEWH and may lead to identification of novel potential drug targets for promoting CEWH.

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References

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